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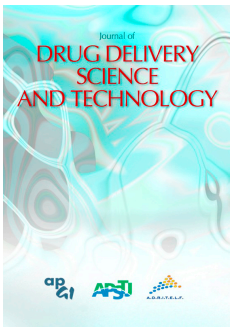

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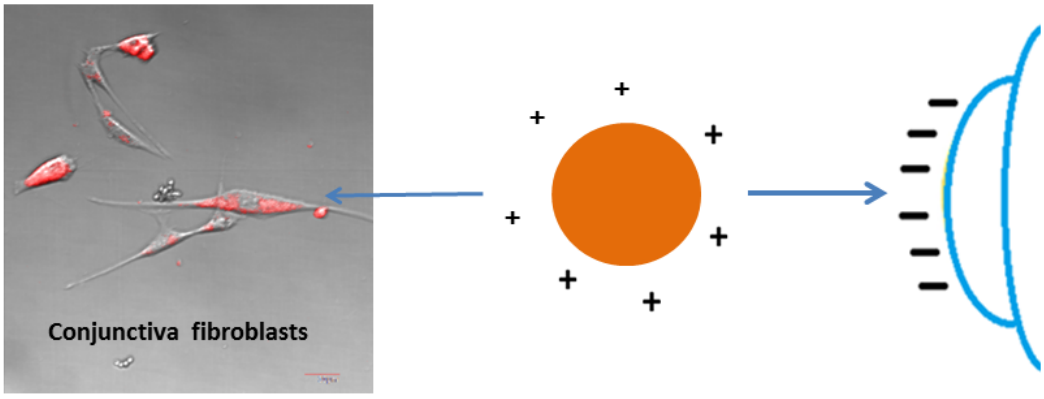
Conjunctiva fibroblasts
Lipid nanoparticle with +ve charge
Eye

Development and evaluation of cationic nanostructured lipid carriers for ophthalmic drug delivery of besifloxacin

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Abstract

Besifloxacin hydrochloride (BSF) is a new ophthalmic antibiotic. However, its low water solubility limits its therapeutic efficacy. This article reported a novel lipid based drug delivery system to enhance ocular bioavailability of BSF. Cationic nanostructured lipid carriers (CNLC) were prepared and optimized by Box-Behnken design of using Design Expert Software®. Effect of concentration of three independent variables, Stabilizer, Solid lipid and Liquid Lipid were studied on three dependent variables Particle size, Polydispersity Index (PDI) and zeta potential of the CNLC particles. The surfactant, hexadecyltrimethylammonium bromide (CTAB) was used to optimize the surface charge of the nano-particles. The BSF loaded nano-particles, CNLC-BSF, were characterized fully. Rhodamine was trapped to CNLC-BSF for imaging. The cytotoxicity and cellular infiltration of CNLC-BSF were assessed by 2-dimensional (2D) and 3-dimensional (3D) conjunctival tissue model. It was revealed that the CNLC's values, particle size, PDI and zeta potential remained stable over 2-week storage. The entrapment efficiency was around 80% for optimum formulation. The cell internalization of CNLC increased when CTAB concentration increased in CNLC-BSF. The formulation showed good penetration property through 3D tissue model. The cytotoxicity assessed by MTT assay showed minimum 60% cell viability on conjunctival fibroblast model for the optimized formula with inclusion of 0.6 mg/mL BSF.

Keywords: cationic nanostructured lipid carrier; ophthalmic drug; bioavailability; infiltration; besifloxacin

1. Introduction

The world is heading towards a post-antimicrobial era in which antibiotics would become inefficient to kill microbes because of 'antimicrobial resistance'. In May 2015 World Health Assembly adopted a global action plan to combat 'antimicrobial resistance' [1]. One of the important objectives of the assembly was 'to optimize the use of antimicrobial medicines in human and animal health'. Bacterial conjunctivitis is an ophthalmic condition arises because of aerobic microbes such as *Staphylococcus aureus*, which needs an antibiotic treatment regimen to reduce the complications and associated chronic conditions. Ocular drug delivery is governed by eye anatomy such as low volume of cul-de-sac and physiology that decrease bioavailability of instilled drugs. The reflex processes like lacrimation and blinking reduce the drug residence time [2].

The activity of Besifloxacin HCl (BSF) is broad spectrum antibiotic which inhibits bacterial enzymes, DNA gyrase and topoisomerase IV. BSF is active against drug-resistant strains such as ciprofloxacin resistant *Staphylococcus aureus* [3]. BSF is practically insoluble in water with log P value of 0.7 [4] Besifloxacin suspension 0.6% w/v for ophthalmic use (Besivance by Bausch & Lomb) is FDA in USA approved formulation for treatment of bacterial conjunctivitis [5]. BSF is the first and only fluoroquinolone exclusive for ophthalmic use [3]. The eye drops formulation is the most commonly prescribed dosage form, but it is shown rapid precorneal drainage hence it requires frequent instillation or high drug concentration in order to maintain required level of drug concentration in ocular fluid. To resolve the problem of maintaining drug concentration in ocular fluid there is a need of the drug delivery system which provides a good retention and sustained ophthalmic delivery of the drug. So,

these problems may be overcome by developing the formulation with good bioadhesion and drug retention property [6].

Number of applications of nanotechnology have been explored to address the challenge for prolonged, targeting and convenient delivery of ocular drugs in high quantity [7,8]. Among them, Solid Lipid Nanoparticles (SLNs) is an attractive technique [9-11]. Using lipid as colloidal carriers developed in the last decade bears advantage over the existing traditional carriers, such as emulsions, liposomes and polymeric nanoparticles. The advantages of SLN of small size, large surface area, high drug loading and the interaction of phases at the interfaces has attracted intensive study of SLN in past years. Cationic nanostructured lipid carrier (CNLC) is a modification of SLNs, which contain liquid lipid and possess positive charge on its surface hence they could adhere to negatively charged surface of conjunctival cells [12-13]. The bioadhesion of nanoparticles could increase ophthalmic retention and minimize precorneal drainage after instillation in the eye. Hence, bioavailability of the drug can be enhanced by formulating CNLC for ophthalmic instillation.

The aim of the current study is to develop antimicrobial loaded cationic nanostructured lipid carrier to enhance ocular bioavailability using BSF as a broad-spectrum antibiotic. The current study describes the statistical technique to optimize BSF loaded CNLC formulation (CNLC-BSF) using Box-Behnken design as a strategy of Quality by Design better known as QbD. Three targeted parameters of the fabricated nanoparticles, particle size, poly dispersity index (PDI) and zeta potential have been selected to develop optimum formulation through quantity and types of solid lipid, liquid lipid and stabilizer. Gelucire 50/13 along with Hexadecyltrimethylammonium bromide CTAB were used to develop CNLC

employing simple melt emulsification method. The optimized formulations were assessed for cytotoxicity and cellular intake capacity, and penetration of the formulation in 2D and 3D conjunctival fibroblast tissue models have been undertaken. The developed tissue models simulate biological condition hence the optimized formulation bears potential of ophthalmic drug delivery system. The nanotechnology-based drug delivery systems developed in this project may be employed after further investigation for treatment of chronic ocular diseases such as conjunctivitis in which frequent drug administration is necessary [14-16].

2. Materials and Methods

2.1. Materials (lipid, stabilizer, surfactant)

Besifloxacin Hydrochloride was purchased from Xi'an Shunyi Bio-Chemical Technology Co. Ltd China, Gelucire® 50/13, Compritol® 888 ATO and Labrafac PG ® were kind gift samples from Gatteffose UK. Hexadecyltrimethylammonium bromide (CTAB), Rhodamine B octadecyl ester perchlorate and Hoechst stain solution were purchased from Sigma Aldrich. Remaining all chemicals were of analytical grade.

2.2 Statistical experimental design

Design-Expert software (Design-Expert 11.1.2.0, State-Ease Inc., Minneapolis, USA) was used to perform statistical calculation and experimental design. Box–Behnken design [8,17,18] was used with 3-factors, 3-levels, and 17 runs for the optimization study. Independent variables were the concentration of Stabilizer (X_1), Solid Lipid (X_2), Liquid Lipid (X_3) while dependent variables were Particle Size (Y_1), Poly Dispersity Index (PDI) (Y_2) and Zeta Potential (Y_3) for optimization of besifloxacin hydrochloride loaded CNLC.

Gelucire 50/13, Compritol 888 ATO and Labrafac PG were selected as the stabilizer, solid lipid and liquid lipid, respectively according to previous experiments. Their concentrations were set at low, medium and high levels based on the results of initial trials. 3-Levels for independent variables were used as listed in Table 1. Objective formulation for the present study was selected for maximizing Zeta Potential while minimizing particle size and PDI. Analysis of variance (ANOVA) was employed for establishing the statistical validation of the polynomial equations generated by Design Expert software. The quadratic model was used for statistical processing as suggested by software. Feasibilities were conducted over the experimental design space to find the compositions of the optimized CNLC formulation. 3-Dimentional response surface plots were generated by the Design Expert software.

2.3. *Method of preparation of Besifloxacin loaded CNLC*

A simple melt emulsification and homogenization method was employed to make drug loaded CNLC [19-21]. Solid lipid (Compritol 888 ATO), Stabilizer (Gelucire 50/13) and Liquid Lipid (Labrafac PG) were transferred to a glass vial which was heated to 85°C. Then drug (besifloxacin) fluorescent dye (Rhodamine B) and CTAB was added to this vial. Distilled water, 9.4 ml was added to the vial and immediately vortexed (approx. 1200 rpm) until it formed the pre-emulsion. The pre-emulsion was transferred to a narrow glass beaker (50 mL) and maintained at 85°C using hot water bath. The pre-emulsion was sonicated using a probe sonicator at 25W, amplitude 70 for 8 min (Fisherbrand Model). The resulting nanoemulsion was cooled to room temperature for solidification. The resultant nanodispersion was passed through cell strainer (40 μm) to remove any gritty particles. The resulted CNLC-BSF was stored in room temperature for further characterization.

2.3.1 Preparation of BSF loaded CNLC with varying amount of CTAB

BSF loaded CNLC formulation was prepared as mentioned in previous section with varying amount of CTAB. Various formulation with concentration of 0, 0.05, 0.1, 0.2, 0.4, 0.8 mg/mL of colloidal dispersions were prepared to find its effect on zeta potential.

2.4 Characterization of CNLC-BSF formulation

2.4.1 Dynamic light scattering (DLS)

2.4.1.1 Particle size and PDI

Dynamic light scattering (DLS) method was used for measurement of particle size of CNLC [22]. Malvern instrument Nano ZS (Malvern, UK), Zetasizer 12 mm square polystyrene disposable cuvettes (DTS0012) were employed for measurement of particle size which was analysed by Zetasizer software by averaging 10 measurements. The sample was 500 times diluted before using for particle size measurement. Sample was equilibrated for 120 seconds and measurement was done at 25°C.

2.4.1.2 Zeta potential

Malvern instrument Nano ZS (Malvern, UK), Folded Capillary Zeta Cell (DTS1070) were employed for measurement of Zeta potential of CNLC through the Zetasizer software [23]. Sample was 500 times diluted then equilibrated for 120 seconds and measurement was done at 25°C by averaging 30 measurements.

2.4.2 TEM

Transmission Electron Microscopy was used to analyze the nanoparticle morphology (JEM-1230 JEOL,

Japan). Dispersed sample was diluted with distilled water to make 0.05% w/v of solids dispersed throughout aqueous medium. 2 µL of diluted sample was spread over 300 mesh copper grid and allowed to dry for 1 hour before imaging [24].

2.4.3 Entrapment Efficiency

Entrapment efficiency of drug was determined using ultracentrifuge filtration [9] indirect method. Besifloxacin loaded nanoparticulate suspension was filled in inner unit of ultra-filter fitted centrifuge tubes (MWCO 10K Amicon ultra-4, Merck Millipore Ltd. IRL) and filtrate was obtained in outer unit of ultrafiltration after centrifugation at 3000 G (Centrifuge Model) for 30 min. Presence of drug (Besifloxacin) in filtrate was determined using plate reader through UV absorbance method at 247 nm wavelength [25]. Percentage of Entrapment Efficiency (%EE) of drug (Besifloxacin) in CNLC was calculated using following formula [26].

%EE = (Initial amount of drug-Amount of free drug present in filtrate) / (Initial amount of drug) ×100

2.4.5 FT-IR

FTIR spectra of the particles were collected using a FT-IR spectrometer (Nicolet iS50, Thermo-Fisher, UK) [27,28]. Air dried sample of CNLC formulation or powder drug (besifloxacin) was suspended in acetone. The suspension was spread over a calcium fluoride window and dried at room temperature. After the acetone evaporated, a thin layer of the samples was formed for the measurement. FTIR spectra were corrected on the baseline; also normalized and smoothed using OMNIC software.

2.5 Cellular study of CNLC

2.5.1 Cell source and conjunctival tissue model

Conjunctival fibroblasts isolated from pig Tenon's capsule + conjunctival tissue were used for all cellular assessment of the NCL particles. The extraction of the cells and formation of 3D Tenon's capsule + conjunctival tissue model followed the protocols established in the lab [29,30]. In brief, porcine eyes were obtained from a local abattoir within a few hours of slaughter. The Tenon's capsule tissue and bulbar conjunctival membrane were dissected out and then digested enzymatically using 3 mg/ml dispase (Sigma-Aldrich, UK) followed by 3 mg/ml collagenase (Sigma-Aldrich, UK). The obtained cells were cultured in high glucose Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal bovine serum, 1% L-glutamine and 1% antibiotics (penicillin & streptomycin) at 37°C, 5% CO_2. The 3D conjunctival tissue model was constructed by incorporating the fibroblasts into 3 mg/ml collagen hydrogels (Fisher Scientific, UK) at a density of 4 x 10^4 cells per 50 µl gel. After the hydrogels were set, the supplemented culture medium was added for culture.

2.5.2 Cytotoxicity study (MTT assay)

MTT assay was performed for cytotoxicity study of Besifloxacin loaded CNLC. Fibroblast cells were seeded in a 96-well plate with the density of 4000 viable cell per well. After the cells were fully attached and stretched, the medium was replaced with 1%, 5% and 10% of besifloxacin suspension (0.6 mg/mL) and two optimum CNLC-BSF formulations containing 0.6 mg/mL of besifloxacin with low (0.2 mg/mL) and high (2.5 mg/mL) concentration levels of CTAB in triplicate [13,27]. After 1 hour of incubation at 37°C, old medium from each well were replaced with the fresh medium containing 5% MTT solution (5 mg/mL in Phosphate buffered saline) and incubated for 2 hours until formazan crystals appeared. The

medium was removed from each well and 120 μL dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. Absorbance DMSO solution was measured at 247 nm by UV detector using microplate reader (BioTek Synergy II). Untreated cells were taken as control and assumed 100% viability and cells without addition of MTT were used as blank for the calibration of spectrophotometer to zero absorbance. Percentage viability was calculated using the following formula [26]:

%Cell viability = (Absorbance of each well) / (Average absorbance of untreated wells) ×100

2.5.3 CTAB concentration in the formula and cell internalization in 2D cell model

To assess the effect of CTAB concentration in the optimum CNLC formulation on cell internalization capacity, five CNLC formulation with variable concentration of CTAB (0 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL and 0.8 mg/mL) were used to culture fibroblast cells using above setting. After 1 hour incubation of the CNLC formulations, fresh culture medium was replaced and cell images were observed and collected using a confocal microscope (Olympus, UK) [29,31].

2.5.4 Cell intake and penetration of NLC in 3D tissue model

After the fibroblast cells in collagen gel became elongated and distributed evenly, the culture medium was replaced with fresh culture medium containing 1%, 5% and 10% of optimum CNLC formulation which was prepared to contain 0.2 mg/mL of CTAB, besifloxacin and Rhodamine B (fluorescent dye). After 1 hour incubation, the CNLC formulation containing media were replaced with normal culture medium. The cell morphology and NLC locations were observed and the images were collected using a confocal microscope [29,31].

2.5.5 Quantification of NLC intake

10^5 conjunctiva fibroblast cells were seeded in T-25 flasks and incubated for 48 hours at 5% CO_2 at 37°C. After that culture media was replaced with fresh culture media containing 5% optimum CNLC formulation containing 0.6 mg/mL of besifloxacin. The flask was incubated for 1 hour then culture medium was removed and washed thrice with PBS. Then cells were passaged, and the cell-pellet was collected. Then 250 µL DMSO was added to cell-pallet to dissolve all cell content along with internalized besifloxacin. The solution was centrifuged at 1200 rpm for 3 min and supernatant DMSO was transferred to 96-well plate to read the absorption at 247 nm in a microplate reader (BioTek Synergy II). Pure DMSO was taken as blank [26]. Concentration of besifloxacin present in DMSO was determined using a besifloxacin calibration curve prepared using known concentration solution of besifloxacin in DMSO. The achieved concentration of BSF in fibroblast cells was more than required minimum inhibitory concentration (MIC_{90}) of BSF i.e. 2 µg/mL [3].

3. Results and discussion

3.1 Optimization of NLC-BSF formulation

The critical independent variables for optimization were identified through literature survey and trial method. The influential parameters were identified to be solid lipid concentration, stabilizer concentration and liquid lipid concentration. The Box Behnken design method (BBD) was used for optimization as it requires fewer runs than other statistical methods. Also, BBD is more efficient in optimization as compare to one or two variable study at a time because BBD is useful to analyze combine effect of different variables on response. The BBD suggested 17 formulations having different combination of concentration of independent variables. The values of three dependent variables, particle

size of NLC, PDI and zeta potential were found in the range of 98.04 to 230.12 nm, 0.144 to 0.351 and 12.83 to 26.65 respectively (Table 2). The 3-Dimentional response graphs as shown in Figure 1 were obtained for Y_1, Y_2 and Y_3 respectively, representing the interaction effects of the factors (X_1, X_2 and X_3) on the responses (Y_1, Y_2 and Y_3). Mathematical expressions for various responses in terms of independent variables were obtained as quadratic equations [8]. Optimum formula for CNLC is as shown in Table 3 and the desirability index of formulation was found to be 0.278 through Design Expert Software.

3.1.1 Effect of independent variables on Particle size

Particle size of NLC formulation need to be lower than 1000 nm for ophthalmic use. Lower particle size favorites stability as the particles remain in Brownian motion which prevent sedimentation [12]. Effect of stabilizer (Gelucire 50/13) on particle size was found to be more prominent where increase in stabilizer concentration led to reduction in particle size (Fig. 1A-A''). This effect may be attributed to PEG present inside gelucire 50/13 which acted as surfactant hence it should be responsible for reduction in interfacial tension [32].

Particle size (Y_1)

$$Y_1 = 238.432 - 14.4091\ X_1 - 0.52225\ X_2 + 5.56062\ X_3 - 0.073875\ X_1 X_2 - 0.103475\ X_1 X_3 + 0.01125\ X_2 X_3 + 0.32192\ X_1^2 + 0.083056\ X_2^2 - 0.035325\ X_3^2$$

3.1.2 Effect of independent variables on PDI

Polydispersity Index (PDI) is a measure of particle size homogeneity which varies from 0 to 1. If the value of PDI is closer to zero, then it indicates higher uniformity or homogeneity and maximum

particles are of nearly same size and size distribution is narrow [33]. The combined effect of stabilizer and solid lipid concentration on PDI was studied using Box Behnken model. It was found that minimum concentration of both variables i.e. stabilizer and solid lipid increased the PDI value. The effect may be attributed to reduction in concentration of total solid substance in formulation and proportional increase in liquid lipid concentration which ultimately reduced the consistency of internal phase (Fig. 1B-B''). Low consistency of internal phase needed comparatively more time for solidification hence it provided enough time for internal phase to orient itself in regular spherical shape under influence of interfacial tension [8]. Also, size reduction become more regular with low consistency of internal phase hence PDI reduces with proportional increase in concentration of liquid lipid (when compared to solid lipid) in formulation.

PDI (Y_2)

Y_2= - 0.260158 + 0.0255812 X_1 + 0.01505 X_2 + 0.0061875 X_3 - 0.00079 X_1 X_2 - 0.0004975 X_1 X_3 + 0.000005 X_2 X_3

3.1.3 Effect of independent variables on Zeta Potential

Also, it was found that increase in stabilizer concentration caused reduction in zeta potential. This may be because of covering/shielding of CTAB by the stabilizer (Fig. 1C-C''). The effect of different CTAB concentration in optimum formulation was then separately studied and it was found that increase in CTAB concentration from 0 mg/mL to 0.8 mg/mL resulted in increase in zeta potential of CNLC from -7.9 mV to +23.9 mV hence the 0.2 mg/mL CTAB concentration was selected as it provides good zeta potential of +16.6 mV.

Zeta potential (Y_3)

$Y_3 = 22.0875 - 1.12325\ X_1 + 1.1955\ X_2 - 0.9785\ X_3 - 0.01475\ X_1\ X_2 - 0.0135\ X_1X_3 - 0.054\ X_2\ X_3 + 0.0318\ X_1^2 + 0.00255\ X_2^2 + 0.0862\ X_3^2$

3.2 Validation of statistical experimental design

The actual experimental values of the responses versus corresponding predicted values plot was generated by Design Expert software for particle size, PDI and zeta potential to obtain scatter plot as shown in Fig. 2a-c whereas Fig. 2a'-c' indicate corresponding run sequence residual plot. The R^2 value for particle size, PDI and zeta potential was found to be 0.9991, 0.9911 and 0.967 respectively. The software generated best fit model for all the three variables was quadratic model with R^2 value or coefficient of correlation nearly equal to unity, the lack of fit F-value for all the three responses were non-significant for quadratic model which indicate good fit, as shown in Table 4. Also, the difference between predicted R^2 and adjusted R^2 was found to be less than 0.2 for all the three responses i.e. Y_1, Y_2 and Y_3 which indicate both the values of R^2 for all responses were in reasonable agreement with each other.

The run sequence residual plot is a scatter plot in which each residual is plotted versus an index i.e. order of making formulation with respect to time. Figure 2a'-c' shows that the residuals from a straight-line fit to the particle size, PDI and zeta potential data plotted in run order. This plot represents good randomized run order. For all these reasons the model can be used to navigate the design space for all the three responses (particle size, PDI and zeta potential) also it is valid for the purpose.

3.3 Characterization of optimum CNLC-BSF formulation

3.3.1 Dynamic light scattering (DLS)

3.3.1.1 Particle size and PDI

DLS technique measures the Brownian motion of suspended particle in liquid medium and correlates it with particle size [6]. Brownian motion of particles is inversely proportional to particle size. The knowledge of viscosity and temperature plays important role in determination of particle size. The measurement was conducted at 25°C when dispersion medium was water. DLS technique measure the translational diffusion coefficient of the particles within the liquid and the particle size of the particle could be determined using Stokes-Einstein's equation in term of z-average diameter, which is average hydrodynamic diameter of the nanoparticles [33]. Higher concentration of solid lipid found to increase particle size of CNLC. Z-average particle size (hydrodynamic diameter) was found to be 173.6 nm with PDI 0.188.

3.3.1.1 Zeta potential

Zeta potential is the potential difference between shear plane and electroneutral region of dispersion medium [34]. Zeta potential measurement has been done by determining particle velocity due to electrophoresis under influence of electric field [6]. The zeta potential was in the range of +12.83 mV to +26.65 mV when a constant concentration of CTAB (0.2 mg/mL) was used while varying the concentration of independent variables (stabilizer, solid lipid and liquid lipid) during optimization of CNLC. The change in zeta potential in CNLC for constant CTAB concentration was attributed to shielding effect of Gelucire 50/13.

3.3.1.2 Effect of CTAB on zeta potential

CTAB induces positive surface charge on CNLCs. This led to increase in positive zeta potential as shown in Figure 3. When the formulation parameters (stabilizer, solid lipid and liquid lipid) for optimum formulation were kept constant and variable concentration of CTAB from 0 mg/mL to 0.8 mg/mL were used it resulted in increase of zeta potential from -7.9 mV to +23.9 mV. The zeta potential for optimum CNLC-BSF with CTAB

concentration of 0.2 mg/mL was found to be +16.6 mV, confirmed through nanozetasizer software. Formulation with a CTAB concentration 0.2 mg/mL showed good zeta potential i.e. more than +16.0 mV.

3.3.2 TEM

Pre-emulsion sample made for optimized CNLC formulation containing BSF and rhodamine B was immediately removed before sonication but after 3 minutes vortex mixing of lipid phase with aqueous phase in warm condition (85°C) and allowed to solidify. The TEM image before sonication (Fig. 4A) reveals larger particle size (> 1000 nm). After sonication TEM image (Fig. 4B) for corresponding CNLC formulation was found to be of small size (<300 nm) with more uniformity and regular spherical shape. The particle size was in accordance with dynamic light scattering analysis results. This study confirmed that proper size reduction and good PDI was achieved because of sonication.

3.3.3 Entrapment efficiency

Entrapment efficiency of CNLC-BSF formulation by ultracentrifuge filtration indirect method was found to be around 80%. This means maximum amount (80%) of BSF was entrapped in CNLC lipid core and 20% was available in aqueous phase for immediate release at the site of action which is the property of good sustained drug delivery system. The formulation was found to be stable for a period two weeks during storage and there was no significant change in entrapment efficiency.

3.3.4 FTIR

FT-IR spectroscopy was performed to investigate any chemical interaction between the encapsulated drug (BSF) and CNLC excipients. Figure 4C shows FTIR spectra of plain CNLC formulation without

drug (a), besifloxacin loaded CNLC formulation (b) and besifloxacin HCl powder (c). In all the three spectra it shows strong peak at 3100 cm^{-1} because of -OH stretching. Strong peak near 1670 cm^{-1} in all the three spectra represented C=O carbonyl stretching. There was no significant difference between spectra of plain CNLC formulation without drug (a), besifloxacin loaded CNLC formulation (b) hence BSF remained physically entrapped inside CNLC without chemically interacting with excipients.

3.4 Cellular interaction of optimum formulation

3.4.1 Cytotoxicity study

Figure 5 reveals that cell viability for besifloxacin suspension through MTT assay was high and reached above 90% when besifloxacin suspension was diluted at 1% and 5% with culture media. For 1% and 5% optimum formulation (containing 0.2 mg/mL of CTAB) added to culture media the cell viability was found to be above 80% while for 1% of optimum formulation (containing 2.5 mg/mL of CTAB) added to culture media the cell viability was found to be above 60%. Results suggest that toxicity of optimum CNLC formulation was CTAB-concentration dependent. Cytotoxicity may have increased because of accumulation of excessive positive charge inside cell due to CTAB as evident from cell internalization study.

3.4.2 CTAB concentration effect on internalization of CNLC in 2D fibroblast cell model

CTAB concentration from 0 mg/mL to 0.8 mg/mL in besifloxacin loaded optimum CNLC formulation demonstrated strong effect on cell intake [35]. Figure 6 shows gradient increase in intensity of fluorescence when laser power of confocal microscope was maintained constant, which revealed that, there was increase in cellular intake of CNLC when CTAB concentration was increased. Cellular intake

maximized with increased in CTAB concentration because of increase in zeta potential (from -7.9 mV to +23.9 mV) which allowed firm adherence to negatively charged cell membranes [12]. In separate experiment the rhodamine and BSF loaded optimum CNLC formulation (containing 0.2 mg/mL CTAB) was selected to investigate cellular internalization using confocal laser microscopy.

3.4.3 Internalization of CNLC formulation using 3D model

Figure 7 shows z-stack images of rhodamine B and BSF loaded CNLC formulation (containing 0.2 mg/mL CTAB) penetrating through 3D conjunctival tissue model. The CNLC formulation have capacity to penetrate collagen gel and fibroblast cells because of its fine size and bio-adhesion property. Even 1% of the formulation in culture medium can penetrate cells within one hour as evident from confocal laser microscopy imaging. Corresponding cell images suggest the CNLC formulation internalized after penetrating through thick collagen gel based conjunctival tissue model. The high bioadhesion of CNLC on conjunctiva facilitated the internalization of CNLC in upper conjunctival cells. We recognize that to allow CNLC for clinical application, more rigorous cytotoxicity test by ex vivo and in vivo models have to be undertaken, their effects on other tissue, e.g. sclera require further investigation.

4. Conclusion

Besifloxacin HCl loaded Cationic Nanostructured Lipid Carriers (CNLC) were developed for conjunctivitis as novel ocular drug delivery system. Design expert Software was employed for statistical optimization of formulation and Box-Behnken design was used for this purpose. Data analysis and processes validation suggested validity of design space because actual values of formulation parameters were very close to predicted values. Characterization of optimum CNLC formulation for various

parameters like particle size, PDI, zeta potential, TEM, FT-IR, entrapment efficiency revealed that the technique of formulation production was robust and bears potential of scale-up. Particle size was in range i.e. 98.04 to 230.12 nm which is suitable for ophthalmic use. Zeta potential was in range from -7.9 mV to +23.9 mV suggesting formulation parameters can be modulated to obtain desired zeta potential and this is crucial for bio-adhesion with negatively charged cell membrane. *In vitro* study of optimum formulation for cytotoxicity and collagen gel penetration suggested that developed formulation was safe and effective for ophthalmic use. The achieved concentration of BSF in conjunctival fibroblast cell model was more than minimum inhibitory concentration (MIC_{90}) of BSF i.e. 2 µg/mL. The simple melt emulsification method employed for CNLC in this study demonstrates great potential for industrial scale-up.

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Author contribution statements

MSB and YY conceived the presented idea. MSB, HO, WN conducted the experiments. MSB took the lead in writing the manuscript. All authors discussed the results and contributed to the final manuscript.

Declarations of interest

None

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Figure captions

Figure 1 A-A'') 3D-response surface plot showing effect of independent variables on particle size of NLC formulation; B-B'') 3D-response surface plot showing effect of independent variables on polydispersity index (PDI) of NLC formulation; C-C'') 3D-response surface plot showing effect of independent variables on zeta potential of NLC formulation.

Figure 2 Scatter plot showing predicted values vs actual experimental values for a) Particle size, b) PDI and c) zeta potential; and run sequence residual scatter plot for a') Particle size, b') PDI and c') zeta potential.

Figure 3 Effect of CTAB concentration on zeta potential of CNLC. The values were expressed by mean ± SD, n = 3.

Figure 4 Characterization of CNLC particles. Representative TEM image of the produced particles in A) pre-emulsion before sonication, B) besifloxacin and rhodamine-B loaded optimum CNLC formulation; and FTIR graphs in C): (i) plain CNLC formulation without BSF; (ii) besifloxacin loaded CNLC formulation and (iii) besifloxacin HCl powder.

Figure 5 MTT assay results. Graph indicating % cell viability when incubating with 1% besifloxacin suspension (0.6 mg/mL) or 1% NLC formulation with 0.2 mg/mL CTAB, or 1% NLC formulation with 2.5 mg/mL CTAB, (n=3).

Figure 6 Confocal scanning laser microscopic images showing the effect of CTAB concentration in fabricating CNLC-BSF particles on the particle intaking in a 2D conjunctival model, manifesting as the increased rhodamine intensity in cells. 5% of the CNLC-BSF particles was added to culture media. a) 0 mg/mL (no CTAB); b) 0.1 mg/mL; c) 0.2 mg/mL; d) 0.4 mg/mL; e) 0.8 mg/mL. Scale bar = 50 μm, (n=3).

Figure 7 Confocal scanning laser microscopic image showing the permeation of CNLC-BSF particles in a 3D conjunctival tissue model. 1 % of CNLC-BSF particles containing 0.2 mg/mL of CTAB was added to culture media. a) the 2D image of particle intaking in the cells with Hoechst dye to stain nucleus (blue), scale bar = 50 μm; b) z-stack image, scale bar = 200 μm. The CNLC-BSF particles were rhodamine labeled (red). (n=3)

Table caption

Table 1 Independent variables (factors) of Box–Behnken design and constraints of dependent variables (responses) for optimization of besifloxacin loaded CNLC

Table 2 Observed responses in Box–Behnken statistical design for development and optimization of besifloxacin loaded CNLC with predicted values generated by Design Expert Software.

Table 3 Optimum formula for BSF-CNLC with 0.2 mg/mL CTAB

Table 4 Statistical model fitting summary report extracted from Design Expert Software.

Table 1: Independent variables (factors) of Box–Behnken design and constraints of dependent variables (responses) for optimization of besifloxacin loaded cationic NLC

Factors	Name	Units	Actual Levels used		
			Low (-1)	Medium (0)	High (+1)
X_1	Stabilizer	mg/mL	10.00	20	30.00
X_2	Solid Lipid	mg/mL	10.00	20	30.00
X_3	Liquid Lipid	µL/mL	10.00	15	20.00
Response			Optimization Goal		
Y_1	Particle size	nm	Minimize		
Y_2	Polydispersity Index (PDI)		Minimize		
Y_3	Zeta Potential	mV	Maximize		

Table 2: Observed responses in Box–Behnken statistical design for development and optimization of besifloxacin loaded cationic NLC with predicted values generated by Design Expert Software.

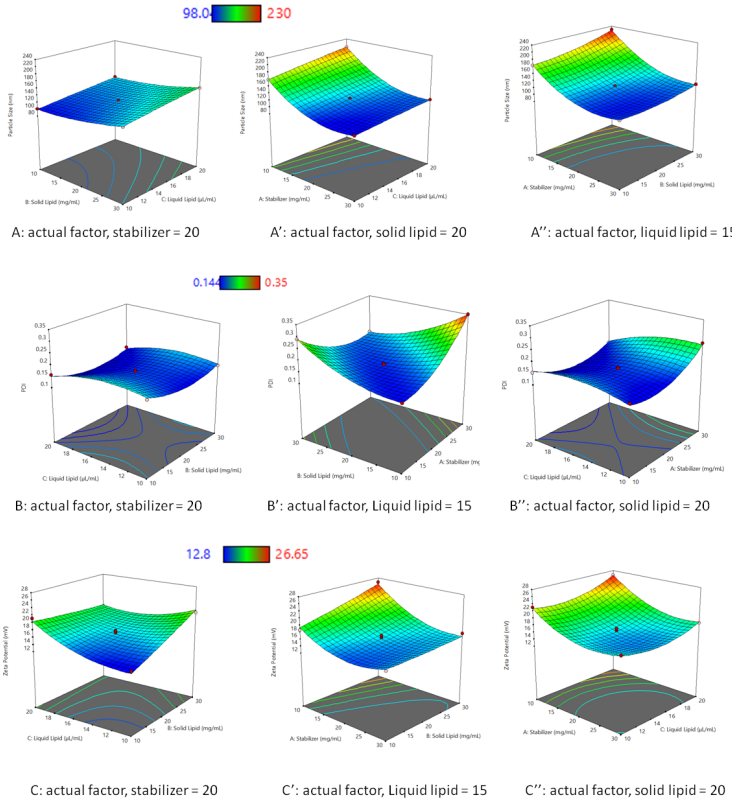
Formulation no.	Run no.	Independent Variables			Dependent Variables					
		Stabilizer (Gelucire 50/13) (mg/mL)	Solid Lipid (Compritol 888ATO) (mg/mL)	Liquid Lipid (Labrafac PG) (µL/mL)	Actual Values			Predicted Values		
					Particle Size (nm)	PDI	Zeta Potential (mV)	Particle Size (nm)	PDI	Zeta Potential (mV)
		X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3
1	6	10	10	15	183.25	0.146	18.61	183.86	0.1456	19.45
2	8	30	10	15	105.85	0.351	14.85	107.42	0.3509	15.32
3	16	10	30	15	230.12	0.290	26.65	228.55	0.2901	26.18
4	12	30	30	15	123.05	0.178	17.21	122.44	0.1784	16.37
5	1	10	20	10	177.99	0.153	23.17	178.52	0.1465	22.80
6	13	30	20	10	98.04	0.250	17.10	97.61	0.2432	17.10
7	4	10	20	20	215.02	0.153	26.62	215.46	0.1598	26.62
8	10	30	20	20	114.35	0.150	18.02	113.82	0.1565	18.39
9	7	20	10	10	100.95	0.181	12.83	99.81	0.1879	12.36
10	17	20	30	10	127.50	0.166	20.78	128.54	0.1724	21.62
11	2	20	10	20	126.31	0.156	21.11	125.27	0.1496	20.27
12	11	20	30	20	155.11	0.144	18.34	156.25	0.1371	18.81
13	5	20	20	15	122.15	0.145	16.56	120.04	0.1534	15.85
14	3	20	20	15	119.05	0.152	16.22	120.04	0.1534	15.85
15	15	20	20	15	118.59	0.161	16.75	120.04	0.1534	15.85
16	14	20	20	15	118.91	0.156	14.14	120.04	0.1534	15.85
17	9	20	20	15	121.49	0.153	15.59	120.04	0.1534	15.85

Table3. Optimum formula for BSF-CNLC with 0.2 mg/mL CTAB

Ingredient	**Quantity**
Drug (BSF)	0.6 mg/mL
Stabilizer	15.08 mg/mL
Solid Lipid	19.85 mg/mL
Liquid Lipid	17.46 µL/mL
CTAB	0.2 mg/mL
Distilled Water	(q.s.)

Table 4: Statistical model fit summary report extracted from Design Expert Software

Response	Models	R^2	Adjusted R^2	Predicted R^2	SD	% C.V.	Adequate Precision	Lack of Fit		Remark
								F-value	p-value	
Y1	**Linier**	0.7942	0.7467	0.6366	19.89	14.34	12.5490	208.52 (significant)	< 0.0001	
	2FI	0.8072	0.6916	0.2902	21.94	15.82	8.6388	292.86 (significant)	< 0.0001	
	Quadratic	**0.9991**	0.9980	0.9923	1.77	1.28	96.3327	**1.34** (non-significant)	0.3790	**Suggested Model**
Y2	**Linier**	0.1337	-0.0662	-0.7432	0.0605	33.34	2.8272	165.81 (significant)	< 0.0001	
	2FI	0.6329	0.4126	-0.5945	0.0449	24.75	7.0900	105.01 (significant)	0.0002	
	Quadratic	**0.9909**	0.9792	0.8881	0.0084	4.65	32.8810	**3.90** (non-significant)	0.1109	**Suggested Model**
Y3	**Linier**	0.5513	0.4477	0.2083	2.96	16.04	7.5321	11.33 (significant)	0.0161	
	2FI	0.7073	0.5317	0.1096	2.73	14.77	7.3114	10.85 (significant)	0.0186	
	Quadratic	**0.9694**	0.9299	0.7541	1.06	5.71	17.6537	**1.08** (non-significant)	0.4526	**Suggested Model**



A: actual factor, stabilizer = 20 A': actual factor, solid lipid = 20 A'': actual factor, liquid lipid = 15

B: actual factor, stabilizer = 20 B': actual factor, Liquid lipid = 15 B'': actual factor, solid lipid = 20

C: actual factor, stabilizer = 20 C': actual factor, Liquid lipid = 15 C'': actual factor, solid lipid = 20

Figure 1 3D-response surface plot showing effect of independent variables on particle size (A-A''); polydispersity index (PDI) (B-B'') and zeta potential (C-C'') of NLC formulation.

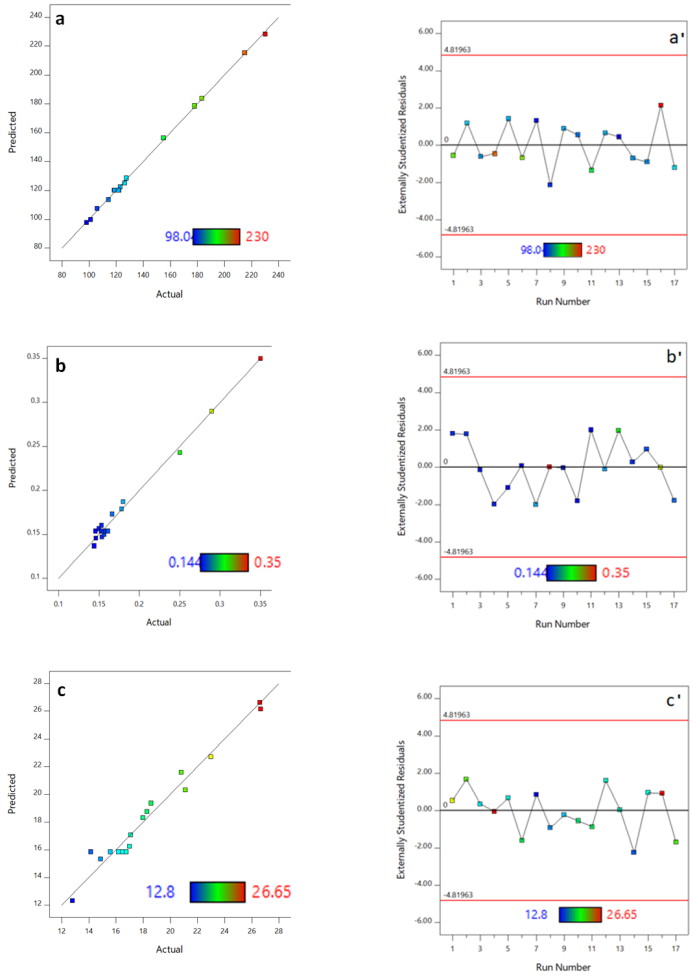


Figure 2 Scatter plot showing predicted values vs actual experimental values for a) Particle size, b) PDI and c) zeta potential; and run sequence residual scatter plot for a') Particle size, b') PDI and c') zeta potential.

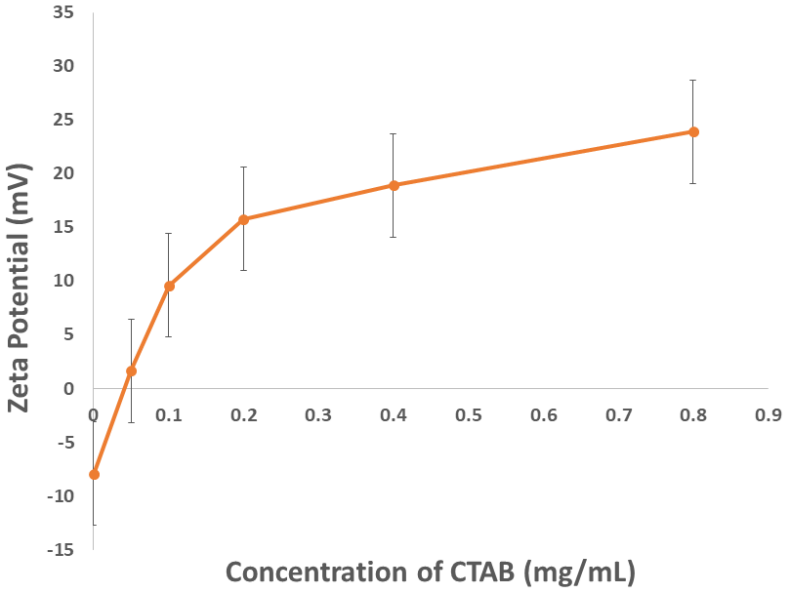

Zeta Potential (mV)
35
30
25
20
15
10
5
0
-5
-10
-15
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Concentration of CTAB (mg/mL)

Fig.3

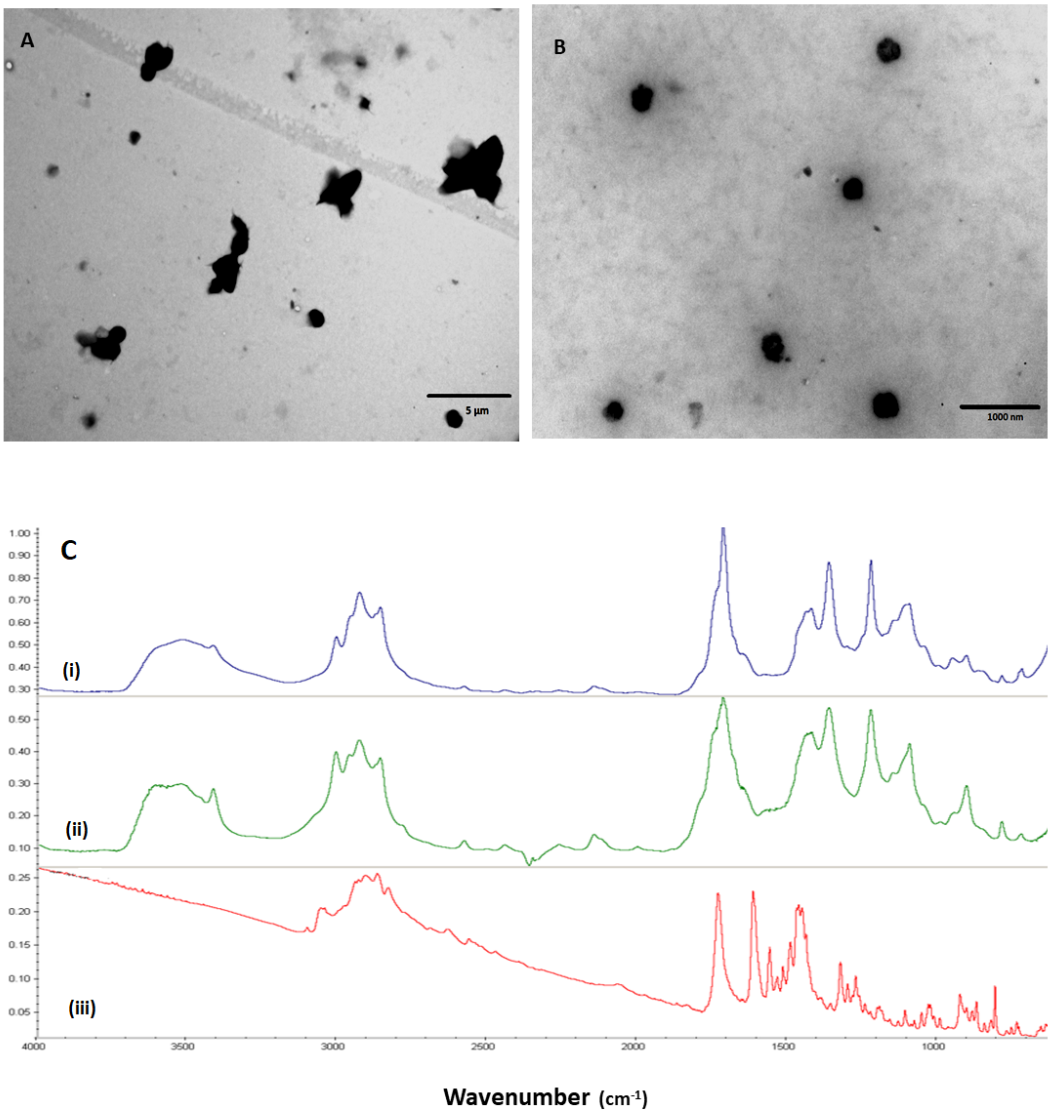


Fig.4

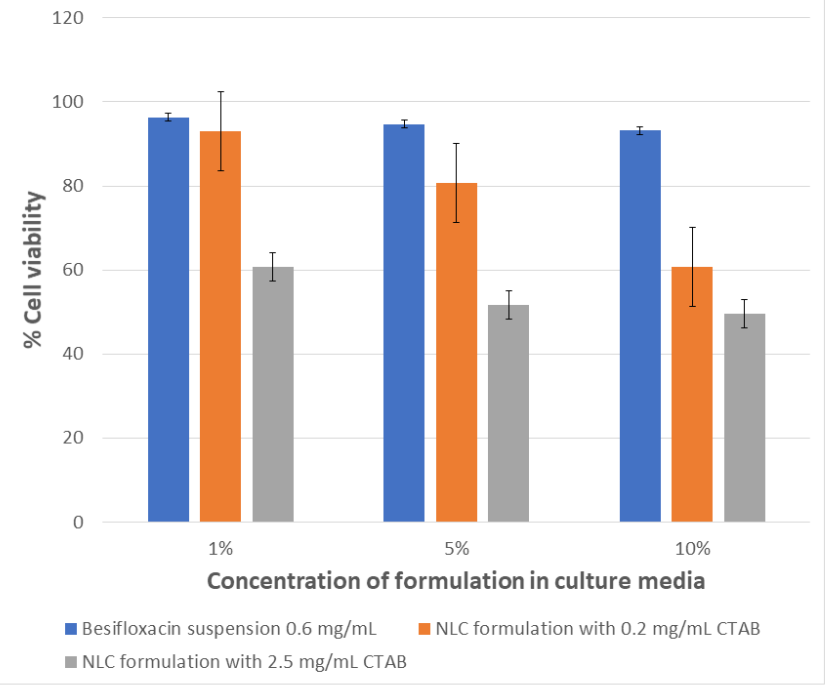

120
100
80
60
40
20
0
% Cell viability
1%
5%
10%
Concentration of formulation in culture media
Besifloxacin suspension 0.6 mg/mL
NLC formulation with 0.2 mg/mL CTAB
NLC formulation with 2.5 mg/mL CTAB

Fig.5

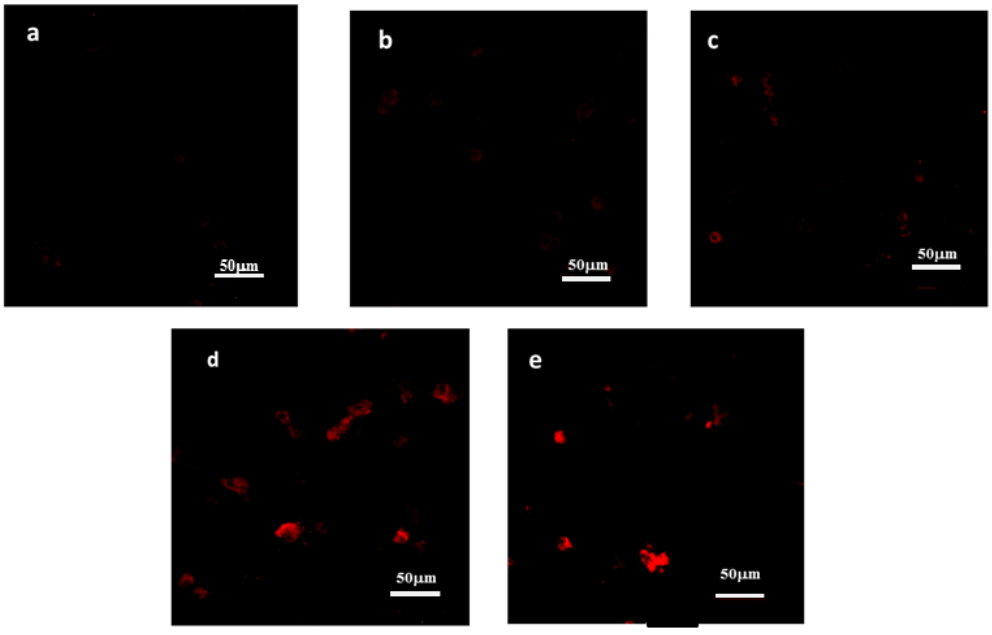


Fig.6

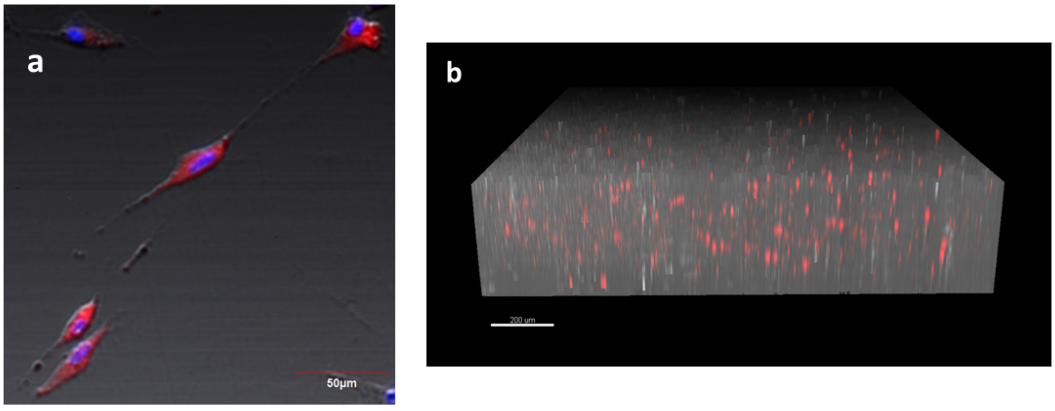


Fig.7

Author contribution statements

MSB and YY conceived the presented idea. MSB, HO, WN conducted the experiments. MSB took the lead in writing the manuscript. All authors discussed the results and contributed to the final manuscript.

Declarations of interest

None